(liters); ml (milliliters); µl (microliters); cm (centimeters); mm (millimeters); µm (micrometers); nm (nanometers); ° C. (degrees Centigrade); h (hours); min (minutes); sec (seconds); msec (milliseconds); Ci (Curies) mCi (milliCuries); µCi (microCuries); TLC (thin layer achromatography); SDS-PAGE (Sodium dodecylsulfate polyacrylamide gel electophoresis); Ts (tosyl); Bn (benzyl); Ph (phenyl); Ms (mesyl); Et (ethyl), Me (methyl).

Example 1

Plasmid Construction and Host Transformation

To study the degradation of SsrA-tagged proteins in *B. subtilis*, variants of pLATIL3 were made in which h-IL3 is expressed with different short peptide tags added to the COOH-terminus of h-IL3:

Variant 1: plasmid pLATIL3-BStag; expresses the hIL3 variant hIL3-AA: hIL3 with an apolar C-terminal *B. subtilis* SsrA-tag [GKTNSFNQNVALAA] (SEQ ID NO:___)

Variant 2: plasmid pLATIL3-DDtag; expresses the hIL3 variant hIL3-DD: hIL3 with a negatively charged C-terminal tag [GKTNSFNQNVALDD] (SEQ ID NO:___), this variant differs from variant 1 (hIL3-AA) only in the last two C-terminal amino acids (two aspartic acids (DD) instead of two alanine (AA) residues)

Variant 3: plasmid pLATIL3-ECtag; expresses the hIL3 variant hIL3-ECAA: hIL3 with an apolar C-terminal *E. coli* SsrA tag [AANDENYALAA] (SEQ ID NO:___)

Plasmids, bacterial strains and media. Table I lists the plasmids and bacterial strains used in this study. E. coli strains were grown in or on 2xYT medium (Bacto tryptone, 16 g/l; yeast extract, 10 g/l; and NaCl, 5 g/l). B. subtilis strains were grown in TSB (Tryptone Soya Broth from Oxoid, 30 g/l), or 2xSSM (Spizizen's minimal medium; Harwood et al. 1990 Molecular

biological methods for Bacillus. John Wiley and Sons, Chichester, United Kingdom), or on SMA (Spizizen's minimal agar; Harwood et al. 1990), or HI-agar (Heart Infusion agar from Difco, 40 g/l). When appropriate, media were supplemented with ampicillin, 100 µg/ml; chloramphenicol, 5 µg/ml; erythromycin, 1 µg/ml; neomycin, 10 µg/ml; spectinomycin, 100 µg/ml; tetracycline, 10 µg/ml and/or isopropyl-β-D-thiogalactopyranoside (IPTG; 500 µM).

Plasmid DNA was isolated with the QIAprep spin miniprep kit (Qiagen) according to the instructions, except that *B. subtilis* cells were incubated with lysozyme (5 mg/ml in buffer P1) for 10' at 37 °C prior to addition of lysis buffer (buffer P2). Chromosomal DNA was isolated as described previously (Harwood et al. 1990). Procedures for DNA restriction, ligation, agarose gel electrophoresis, and transformation of *E. coli* were carried out as described in Sambrook *et al.* (1989. Molecular Cloning: A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). Enzymes were from Life technologies.

Transformation of competent cells was used to transfer DNA (plasmids, linear DNA) into B. subtilis (Harwood et al. 1990). PCR (polymerase chain reaction) was carried out with High Fidelity Platinum Taq DNA Polymerase (Life technologies) and if required PCR fragments were purified with the Qiaquick PCR purification kit (Qiagen). DNA primers were from Life technologies and DNA sequencing was performed by BaseClear (Leiden, The Netherlands). Plasmid pLATIL3TERM was obtained by PCR on pLATIL3 with the primers pLATIL3SXHfw (5' GTC GAC CTC GAG ACC CCA AGC TTG GCG TAA TC 3') (SEQ ID NO:___) and pLATIL3T3rv (5' GTC GAC CTC GAG CGG CAG AAT CTT TTT TTG ATT CTG CCG CAA AGT CGT CTG TTG AGC CTG 3') (SEQ ID NO:___). The resulting DNA fragment was purified, digested with XhoI, self-ligated, and transformed directly into B. subtilis. One of the plasmid clones, found to be correct by DNA sequencing, was designated pLATIL3TERM. This plasmid holds the transcription

terminator of the folC gene (present in primer pLATIL3T3rv) at the 3'end of the AmyL-hIL-3 gene, just in front of an in-frame stop codon. Plasmid pLATILBStag was obtained by a PCR on pLATIL3 with the primers pLATIL3T2FW (5' CTG CAG CTC GAG GAT ATC GTC GAC CGG CAG AAT CAA AAA AAG ATT CTG CCG ACC CCA AGC TTG GCG TAA TC 3') (SEQ ID NO:___) and pIL3BStagRV (5' CTT CTA CTC GAG TCA GGC AGC TAA TGC TAC GTT TTG GTT AAA ACT GTT AGT TTT GCC TGC GCT CAA AGT CGT CTG TTG AGC 3') (SEQ ID NO:___). The resulting PCR fragment was purified, digested with XhoI, self-ligated, and transformed into B. subtilis. A few clones were checked by DNA sequencing and one correct clone was selected and named pLATIL3BStag. Plasmid pLATIL3DDtag and pLATIL3ECtag were made in the same way but instead of primer pIL3BstagRV, primer pIL3DDtagRV (5' CTT CTA CTC GAG TCA GTC GTC TAA TGC TAC GTT TTG GTT AAA ACT GTT AGT TTT GCC TGC GCT CAA AGT CGT CTG TTG AGC 3') (SEQ ID NO:___) and primer pIL3EctagRV (5' CTT CTA CTC GAG TCA AGC TGC TAA AGC GTA GTT TTC GTC GTT TGC TGC GCT CAA AGT CGT CTG TTG AGC 3') (SEQ ID NO:___) were used, respectively. To construct B. subtilis ΔssrA mutants, ssrA and its flanking regions (approximately 2.2 kb) was amplified by PCR with the primers pSsrAFW (5' CAG CTC CGT CTG AGG AAA AAG 3') (SEQ ID NO:___) and pSsrARV (5' CGA AGT GGG CGA TTT CTT CCG 3') (SEQ ID NO:___) and cloned into pCR2.1-TOPO, resulting in plasmid pTPSsrA. Plasmid pSsrASp was obtained by inserting a pDG1726-derived Sp resistance marker (Guérout-Fluery et al. 1995. Antibiotic-resistance cassettes for Bacillus subtilis. Gene 167:335-336) into the unique SacI site in the ssrA gene of pTPSsrA. Finally, B. subtilis 168 ΔssrA and WB600 ΔssrA were obtained by a double cross-over recombination event between the disrupted ssrA gene of pSsrASp and the chromosomal ssrA gene in B. subtilis 168 and WB600, respectively. $SsrA^{DD}$ expressing B. subtilis strains were made as follows: a fragment consisting of a 5' end part of ssrA including the ssrA